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cis Sequences Involved in Modulating Expression of *Bacillus licheniformis amyL* in *Bacillus subtilis*: Effect of Sporulation Mutations and Catabolite Repression Resistance Mutations on Expression

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Nutrient conditions which trigger sporulation also activate expression of the *Bacillus licheniformis* α-amylase gene, *amyL*. Glucose represses both spore formation and expression of *amyL*. A fusion was constructed between the *B. licheniformis* α-amylase regulatory and 5′ upstream sequences (*amyRi*) and the *Escherichia coli lacZ* structural gene to identify sequences involved in mediating temporal activation and catabolite repression of the *amyL* gene in *Bacillus subtilis*. *amyRi*-directed expression in a variety of genetic backgrounds and under different growth conditions was investigated. A 108-base-pair sequence containing an inverted repeat sequence, ribosome-binding site, and 26 codons of the structural gene was sufficient to mediate catabolite repression of *amyL*. *spo0* mutations (*spo0A*, *spo0B*, *spo0E*, and *spo0H*) had no significant effect on temporal activation of the gene fusion when the recipient strains were grown in nonrepressing medium. However, in glucose-grown cultures the presence of a *spo0A* mutation resulted in more severe repression of *amyRi-lacZ*. In contrast, a *spo0H* mutation reduced the repressive effect of glucose on *amyRi-lacZ* expression. The *spo0A* effect was relieved by an *abrB* mutation. Initiation of sporulation is not a prerequisite for either temporal activation or derepression of α-amylase synthesis. Mutations causing resistance to catabolite repression in *B. subtilis* GLU-47, SF33, WLN30, and WLN104 also relieved catabolite repression of *amyRi-lacZ*.

The *Bacillus licheniformis* α-amylase gene, *amyL*, is temporally expressed and subject to catabolite repression both in its natural host and when cloned in *Bacillus subtilis* (16). Catabolite repression is mediated at the level of transcription by sequences downstream from the promoter of *amyL* (16). In this report, the construction of a gene fusion of *B. licheniformis* α-amylase regulatory sequences to the *Escherichia coli lacZ* structural gene (*amyRi-lacZ*) is described. This fusion was used to determine more precisely the sequences necessary for mediating both temporal activation and catabolite repression of *amyL*.

amyL is temporally activated at the onset of the stationary phase under nonrepressing growth conditions, when cells presumably initiate sporulation. This suggests that the two processes are mechanistically related. *spo0* mutations block sporulation prior to its earliest morphological event. They also inhibit the production of proteases (5, 8, 9, 29) and other stationary-phase-associated proteins such as phosphatases and extracellular antibiotics (12, 27). It has now become evident that *spo0A* regulates not only sporulation-associated genes but also a variety of other genes (13, 18, 19). Spo0A inhibits expression of *hpr* and *abrB*. Both of these genes appear to encode negative regulators which regulate expression of genes that are activated postexponentially in response to nutrient starvation (25, 26, 36). *spo0A* is a positive regulator of *spo0H* (4, 7). *spo0H* (*sigH*) encodes a minor vegetative sigma factor, σ^H (4, 7). Expression of *spo0H* in strains carrying an *abrB* mutation is *spo0A* independent (7). The data suggest that Spo0A has an important role as a sensory regulator in determining the regulation of global processes in the cell. Accordingly, it is of interest to study the effects of *spo0A*, *abrB*, *spo0H*, and other *spo0* mutations on *amyRi*-directed expression.

To determine whether the mechanisms of catabolite repression of different enzyme systems share common regulatory pathways, we transformed the *amyRi-lacZ* gene into a *B. subtilis* mutant (GLU-47) resistant to catabolite repression in sporulation (28, 31, 32) and into three strains (SF33, WLN30, WLN104) which exhibit derepressed synthesis of certain enzymes normally subject to catabolite repression (10, 11; W. L. Nicholson, Ph.D. thesis, University of Wisconsin, Madison, 1987). The expression of the gene fusion in these strains grown in repressing (1% glucose) and nonrepressing (no glucose) media was examined.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are listed in Table 1. Strain SO113 is the amylase-negative strain used for the initial cloning of *amyL*. Strain JH642 is the parent strain from which the *spo0* mutations were isolated.

Protoplast transformation. Strain JH648 is not naturally competent; therefore, protoplasts were made as described previously (2).

Plasmid isolation. *B. subtilis* minipreparations were prepared as described previously (33), and *E. coli* minipreparations were prepared by the method of Birnboim and Doly (1). Large-scale plasmid DNAs were prepared similarly except that the DNA was further purified by CsCl-ethidium bromide density gradient centrifugation (20).

DNA manipulations. Restriction enzymes, T4 DNA ligase, Klenow fragment, calf intestinal phosphatase, and polynucleotide kinase were obtained from Boehringer Mannheim

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TABLE 1. *B. subtilis* strains and plasmids used in this study

Strain or plasmid	Genotype or marker	Source
SO113	*trpC2 amyE*	S. A. Ortlepp
JH642	*pheA1 trpC2*	BGSC[a]
JH646	*pheA1 trpC2 spo0A12*	BGSC
JH648	*pheA1 trpC2 spo0B136*	BGSC
JH647	*pheA1 trpC2 spo0E11*	BGSC
JH651	*pheA1 trpC2 spo0H81*	BGSC
ZB449	*trpC2 pheA1 abrB703* SPβ (con)	P. Zuber
ZB369	*trpC2 pheA1 spo0AΔ204 abrB703*	P. Zuber
GLU-47	*strA crsA47*	BGSC
SF32	*gltA292 trpC2 metC3 hutC1*	BGSC
SF33	*gltA292 trpC2 metC3 hutC1 cdh-3*	BGSC
WLN30	*gra-26*::Tn*917*	G. Chambliss
WLN104	*gra-46*::Tn*917*	G. Chambliss
pSL3	Ap^r *amyRi*	M. Stephens
pKD10	Em^r Ap^r; *lacZ* structural gene	H. Wood, K. Devine
pDE37	Cm^r; pBAA1 origin of replication	K. Devine
pRB1	Em^r Ap^r *amyRi-lacZ*	This work
pBL4	Em^r Ap^r *amyRi-lacZ*	This work
pBL12	Em^r Ap^r Cm^r *amyRi-lacZ*	This work

[a] *Bacillus* Genetic Stock Center.

Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. DNA fragments for ligation reactions were isolated by sucrose (5 to 20% [wt/vol]) density centrifugation at 26,000 rpm in a Beckman SW28 rotor at 15°C for 16 to 20 h.

DNA sequencing. The junction of the gene fusion was sequenced on one strand by the Maxam and Gilbert (22) sequencing protocol. A 200-base-pair (bp) fragment was isolated which spans from the *Nde*I site, 16 bp upstream from the *B. licheniformis* α-amylase ribosome-binding site, down to the *Pvu*II site, 50 bp downstream from the 17th codon of the *lacZ* structural gene, and was labeled at the 5′ *Nde*I site.

β-galactosidase assays. Overnight culture (1 ml) was inoculated into 100 ml of LB plus antibiotic, and 1 ml was inoculated into 100 ml of LB plus 1% glucose plus antibiotic. The cultures were vigorously aerated at 37°C, and growth was monitored with a Klett-Summerson colorimeter (filtered with a red filter, no. 66). At different stages throughout the growth cycle, three 1-ml samples were removed and the cells were pelleted by centrifugation in an Eppendorf centrifuge for 30 s and quickly frozen and stored at −20°C. The cell pellets were suspended in 1 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol) containing 40 μg of lysozyme and incubated at 37°C for 15 min. A 0.1% final volume of Triton X-100 was then added to the samples, and lysis was allowed to occur at room temperature for 5 min. Samples were then assayed for β-galactosidase activity by the method of Miller (23). The amount of cellular protein present in the lysed culture was measured by a microassay (Bio-Rad Laboratories, Richmond, Calif.). β-Galactosidase units were defined per milliliter of culture per milligram of protein.

Plate test. β-Galactosidase activity was detected with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) or by spraying the overnight colonies with MUG (4-methylumbelliferyl-β-galactoside) and observing fluorescence under long-wave UV light.

Construction of *amyRi-lacZ* translation fusion in *E. coli*. pKD10 (gift from H. Wood and K. Devine) carries the *lacZ* gene. It was constructed by ligating the *lacZ* gene and Em^r marker (*Sma*I-*Kpn*I fragment) of pTV32 to the *Hin*dII-*Pvu*II origin and Ap^r fragment of pBR322. The first 16 codons of *lacZ* are absent, but this does not effect expression of the gene. Immediately upstream from codon 17 are *Sal*I, *Bam*HI, and *Eco*RI sites. The plasmid was digested with *Bam*HI and *Sal*I to remove the 71 bases homologous with *spoVG* (35), the ends were filled in with Klenow enzyme, *Xba*I linkers were added, and the plasmid was religated with T4 DNA ligase. The addition of *Xba*I linkers created two *Sal*I sites on either side of the *Xba*I site. It was more convenient to have a *Hin*dIII site present upstream from the *lacZ* gene; therefore, the plasmid was digested with *Xba*I and filled in, and a *Hin*dIII linker was ligated to the blunt ends. The plasmid, pKD10-1, was then digested with an excess of *Hin*dIII enzyme to remove multiple linkers and religated. pKD10-1 therefore carries an *Eco*RI site, two *Sal*I sites, and a *Hin*dIII site upstream from codon 17 of the *lacZ* gene (Fig. 1). A 1.1-kilobase *Eco*RI-*Hin*dIII fragment from pSL3 contains the *B. licheniformis* FDO2 α-amylase regulatory sequences including its promoter, ribosome-binding site, ATG initiation codon, and 26 codons of the structural gene. This fragment (referred to as *amyRi*) also contains sequences 5′ to the gene. *amyRi* was ligated to *Eco*RI-*Hin*dIII-digested pKD10-1 and transformed into *E. coli*. The resultant transformants were screened for β-galactosidase activity, but pale blue colonies were only detected after 48 h of incubation at 37°C. Sequence analysis revealed that a CT dinucleotide which should be present at the filled-in *Xba*I site was absent, so that the fusion was out of phase. A *Bam*HI linker was inserted into the *Hin*dIII site, and the plasmid was digested with excess *Bam*HI enzyme, religated, and transformed into *E. coli*. Some of the Ap^r transformants turned dark blue overnight on LB plus X-gal plates. Restriction digest analysis of plasmid DNA prepared from these transformants showed that there is a *Bam*HI site present on the plasmid, designated pRB1 (Fig. 1).

RESULTS AND DISCUSSION

Expression of *amyRi-lacZ* in *B. subtilis* SO113. To define the *cis*-acting sequences involved in modulating expression of the *B. licheniformis amyL* gene, we created a fusion between a 1.1-kilobase *B. licheniformis* DNA fragment and the *E. coli lacZ* structural gene (see Materials and Methods). The *lacZ* structural gene was transcribed and translated from the *amyL* regulatory sequence, *amyRi*. The construct carried 26 codons of the *amyL* gene fused to codon 17 of the *lacZ* gene. The *B. licheniformis* fragment also contained a 393-bp open reading frame immediately preceding *amyL*; sequences upstream, including this open reading frame, are known to be dispensible for *amyL* expression and do not effect catabolite repression of *amyL* (16).

The expression of *amyRi-lacZ* on a multicopy plasmid in *B. subtilis* was examined. pDE37 carries a *B. subtilis* origin of replication on a 3.7-kilobase *Eco*RI fragment from a cryptic plasmid, pBAA1, and a *cat* (chloramphenicol acetyltransferase) gene for selection in *B. subtilis* (gift from K. Devine). This plasmid was digested with *Eco*RI, ligated to *Eco*RI-linearized pRB1, and transformed into *B. subtilis* SO113, selecting for Em^r (5 μg/ml). Some of the transformants carried only the 3.7-kilobase origin fragment ligated to pRB1 (pBL4), while others also carried the Cm^r marker (pBL12) (Fig. 1). Blue colonies were detectable on LB plus X-gal after 40 to 48 h. The center of the colony had a dark blue color which decreased in intensity to pale blue at the

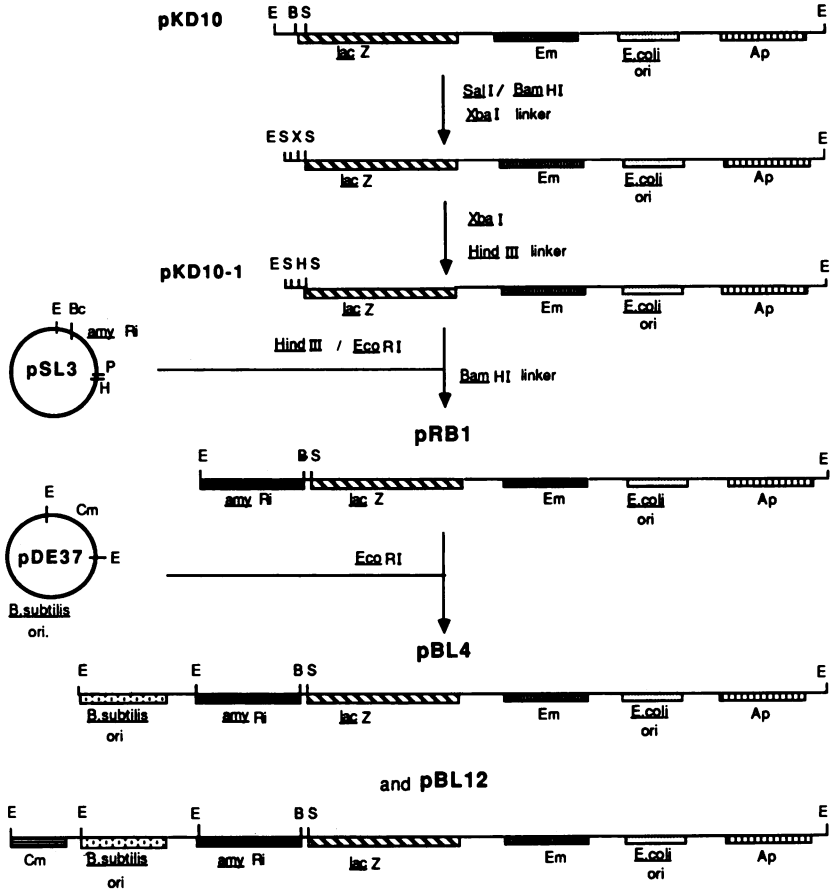

FIG. 1. Construction of *E. coli* (pRB1) and *B. subtilis* (pBL4 and pBL12) plasmids carrying the gene fusion *amyRi-lacZ* (see Materials and Methods and Results sections for details). B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. Ap, Ampicillin resistance marker; Cm, chloramphenicol resistance marker; Em, erythromycin resistance marker. *ori*, Origin of replication. *amyRi*, *B. licheniformis* α-amylase regulatory and 5′ upstream sequences. *lacZ*, *E. coli lacZ* structural gene.

edge of the colony. This suggested that the older stationary-phase cells in the center of the colony are maximally producing β-galactosidase, whereas synthesis of the gene fusion product is low in exponentially growing cells. β-Galactosidase activity was quantified in *B. subtilis* SO113 (pBL4) grown in LB in the presence or absence of 1% glucose. The culture samples were assayed for β-galactosidase activity throughout the growth cycle (Fig. 2). β-Galactosidase accumulated rapidly after the cells entered the stationary phase. The presence of glucose in the growth medium resulted in 8- to 10-fold repression of β-galactosidase activity. The *amyRi-lacZ* gene fusion was catabolite repressed when present on a multicopy plasmid in *B. subtilis*.

To study the expression of the gene fusion in single copy, we integrated it into the *B. subtilis* chromosome. *B. subtilis* SO113 chromosomal DNA was digested with *Eco*RI, ligated to *Eco*RI-linearized pRB1, and transformed into *B. subtilis* SO113. pRB1 carries an *E. coli* origin of replication and cannot replicate autonomously in *B. subtilis*. Only transformants in which the plasmid has integrated into the chromosome by homologous recombination between the chromosomal DNA on the plasmid and the recipient chromosomal DNA will be Em^r. Transformants were selected on medium containing 2 μg of erythromycin per ml and screened for β-galactosidase activity. Pale blue colonies were detected after 48 h of incubation on LB plus X-gal plates at 37°C. From the plate test, it appeared that all the transformants expressed the gene fusion maximally in the stationary phase and showed repressed levels of β-galactosidase when plated on repressing medium (0.5 glucose). A randomly chosen transformant, LC1, was assayed for β-galactosidase activity in liquid cultures (LB and LB containing 1% glucose). *amyRi-lacZ* expression in this transformant was both temporally activated at the onset of stationary phase and subject to glucose-mediated repression (data not shown).

The *B. licheniformis amyRi* sequence directs the synthesis of β-galactosidase in a manner similar to the regulated expression of its cognate structural gene, *amyL*, in *B. subtilis* (16). *amyRi* must therefore contain the sequences essential for mediating both temporal activation and catabolite repression. The promoterless *amyL* gene is still subject to catabolite repression whether it is activated by a plasmid promoter or read from a variety of *B. subtilis* chromosomal promoters (16). Thus, the *cis* sequences essential for mediating catabolite repression of *amyL* in *B. subtilis* are not contained within the amylase promoter or within the structural gene encoding the mature protein or in any sequences 3′ to the gene, but lie downstream from the promoter region and upstream from the signal sequence cleavage site. This sequence is 108 bp long and includes an inverted repeat sequence, TGTTTCAC-20 bp-ATGAAACA (16). Deletions into the left-hand inverted repeat sequence, which lies just 5′ to the putative ribosome-binding site, either abolished activ-

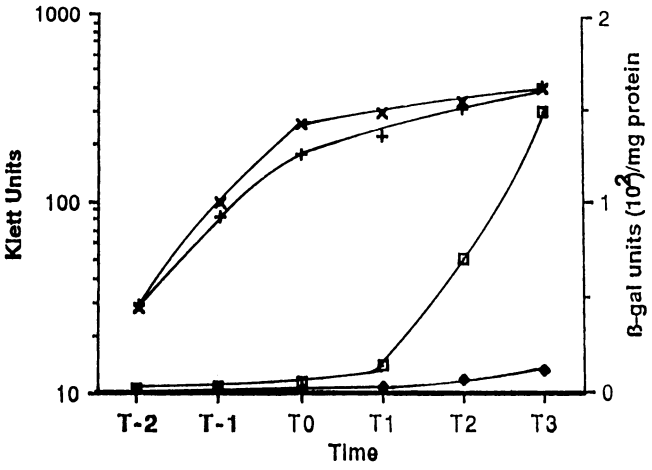

FIG. 2. Growth curves (+, LB; ×, LB plus 1% glucose) and corresponding β-galactosidase activity profiles (⊡, LB culture supernatants; ◆, LB plus 1% glucose culture supernatants) of *B. subtilis* SO113(pBL4). T_0, Time at which cells enter stationary phase. T_1 and T_2 are 1 and 2 h before T_0, respectively. T_1, T_2, and T_3 are 1, 2, and 3 h after T_0, respectively.

ity or altered expression without affecting catabolite repression of *amyL* (data not shown). It will be necessary to carry out site-specific mutagenesis to determine the role, if any, of this sequence. What is clear is that this 108-bp fragment is sufficient to regulate expression of a heterologous gene so that the gene is subject to glucose-mediated repression in the same manner as the *amyL* structural gene.

Expression of *amyRi-lacZ* in *spo0* backgrounds. *spo0* mutations arrest sporulation prior to its earliest morphological stage (stage 0), that is, the formation of an asymmetric septum. *spo0* genes are believed to be responsible for sensing nutritional signals and initiating response to environmental and nutrient conditions (18, 19). It is known that *spo0* genes are also required for the development of competence, for antibiotic production (12), and for the full expression of genes encoding the major proteases (5, 8, 9, 29). We tested for an effect of *spo0* mutations on *amyRi-lacZ* expression. Strains harboring *spo0A*, *spo0B*, *spo0E*, or *spo0H* mutations were transformed with pBL12, which carries the *amyRi-lacZ* fusion (Fig. 1). Strains were grown in nonrepressing (no glucose) or repressing (1% glucose) media and assayed for intracellular β-galactosidase activity. Strain JH642 is the parent strain from which the *spo0* mutations were isolated, and this strain was also transformed with pBL12. It is similar to SO113(pBL4). Synthesis of β-galactosidase occurred maximally at the end of exponential growth and was subject to 10-fold repression in glucose-containing cultures (Fig. 3).

When strains were grown under nonrepressing conditions, none of the *spo0* mutations affected temporal activation appreciably, though the *spo0E*-bearing strain [JH647 (pBL12)] had somewhat higher levels of β-galactosidase activity during vegetative growth than the isogenic Spo$^+$ strain, JH642(pBL12) (Fig. 3).

When grown in the presence of 1% glucose, JH648(pBL12), which carries a *spo0B* mutation, had low enzyme activity levels in the late stationary phase, two- to threefold lower than the repressed levels in Spo$^+$ cultures (Fig. 3). It appears that the *spo0B* mutation exerts some effect on the expression of *amyRi-lacZ* in repressing medium. The *spo0E* mutation, however, had no significant effect on *amyRi*-directed expression in medium containing glucose (Fig. 3); repression in a *spo0E* background was unchanged from wild type. The gene products of *spo0B* and *spo0E* have not been identified. *spo0B* appears to have a vegetative function (3), while *spo0E* is induced before the onset of the stationary phase (24). These genes may be involved, along with the *spo0F* gene product, in the conversion of the *spo0A* gene product from an inactive to an active form (24).

The *spo0H*-bearing strain [JH651(pBL12)] was poorly repressed. As the cells entered the stationary phase, specific activity increased in a manner similar to the activity profile of cultures grown in the absence of glucose (Fig. 3). Repression by glucose was only two- to fourfold. Spo0H has been identified as a sigma factor, σ^H (4, 7). It is possible that the *spo0H* mutation prevents the transcription of a negative factor. The putative gene encoding this factor could have two promoters, a σ^H promoter sequence and a promoter recognized by an alternate form of sigma factor, which would explain the low levels of glucose-mediated repression observed.

In contrast to JH651(pBL12), β-galactosidase activity in a *spo0A*-bearing mutant, JH646(pBL12), was repressed to a greater extent than in the Spo$^+$ parent strain. The presence of glucose in the growth medium caused a severe and permanent repression of *amyRi-lacZ* expression—40- to 50-fold in the stationary phase. There was consistently fivefold-more repression of *amyRi-lacZ* expression in glucose-grown cultures of JH646(pBL12) compared with the Spo$^+$ parent strain (Fig. 3). *spo0A* is the most pleiotropic of all the *spo0* mutations and has been shown to be the site of suppressor mutations that allow sporulation to occur in the presence of defective *spo0B*, *spo0E*, and *spo0F* genes (24). Perego and Hoch (24) suggested that the *spo0A* gene product acts as a transcriptional factor that interacts with RNA polymerase molecules containing minor forms of sigma factors. The reason for increased glucose-mediated repression of *amyRi-lacZ* expression in a *spo0A* background is not clear. It is possible that the absence of the *spo0A* gene product results in the failure of the cell to accurately sense its nutritional environment and consequently in its failure to respond in a manner similar to that of the wild-type strain under glucose-repressing growth conditions. A *spo0A abrB* double mutation relieves many *spo0A* effects, although these mutants are still sporulation negative (36). The *spo0A* gene has recently been shown to repress transcription of the *abrB* gene (26). *abrB* appears to encode a regulator involved in the control of a number of genes whose products are produced at the end of exponential growth. The *abrB* mutation relieved the severe repression of *amyRi-lacZ* exerted by *spo0A* and restored repression to Spo$^+$ levels (Fig. 3).

Strains carrying *spo0* mutations are defective in sporulation initiation. The *spo0*-bearing mutants tested had β-galactosidase enzyme activity profiles similar to that of the Spo$^+$ parent strain when the cultures were grown in nonrepressing medium. This indicates that the initiation of spore formation is not a prerequisite for temporal activation of *amyL*.

***amyRi-lacZ* expression in catabolite repression resistance mutants of *B. subtilis*.** Both sporulation and extracellular α-amylase synthesis are subject to catabolite repression. When glucose, or another easily metabolizable carbon source, is present in the growth medium, *B. subtilis* does not undergo sporulation. Moreover, expression of a large number of enzymes is repressed. This raises the question of whether there are steps common to the mechanism of catabolite repression of sporulation and of enzyme synthe-

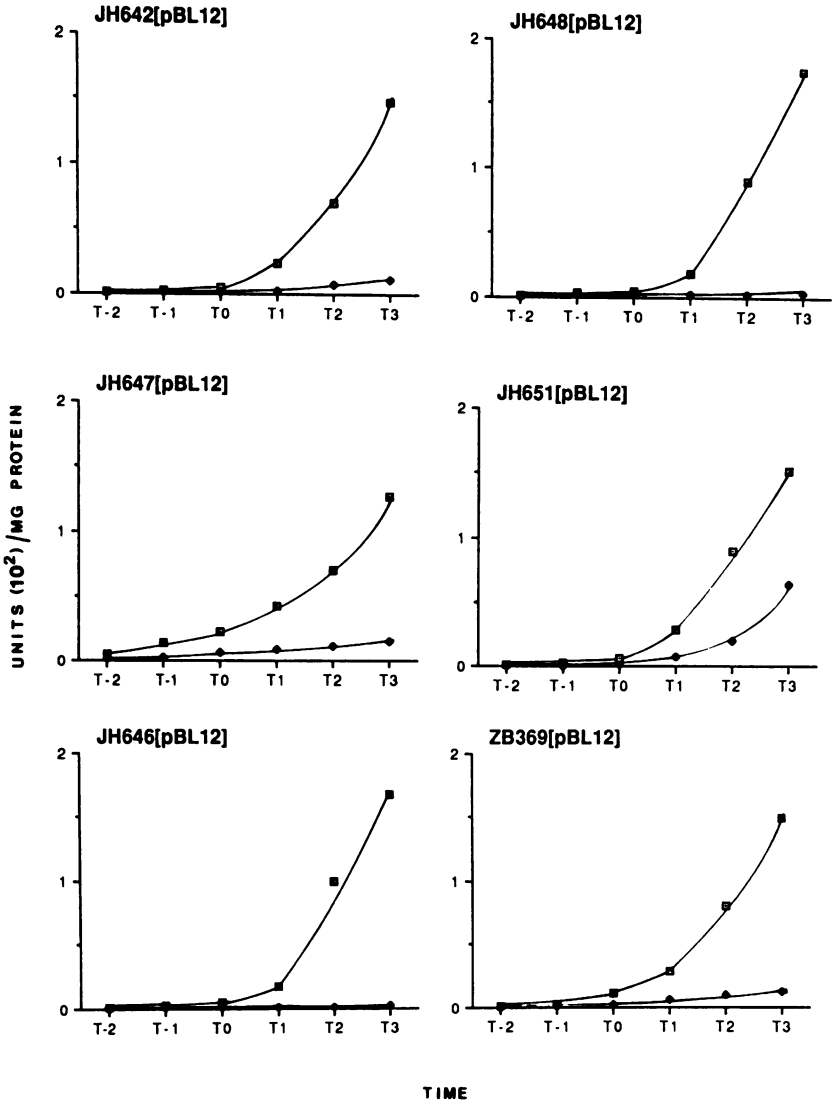

FIG. 3. β-Galactosidase activity profiles of Spo$^+$ strain JH642 and of sporulation-negative strains JH648 (*spo0B*), JH647 (*spo0E*), JH651 (*spo0H*), and JH646 (*spo0A*) and of ZB369 (*spo0A abrB*), all harboring plasmid pBL12. Symbols and *T* values are defined in the legend to Fig. 2.

sis. Mutations have been isolated which allow sporulation to occur in the presence of glucose but which do not derepress enzyme synthesis (6, 14), and conversely there are mutants which are catabolite repression resistant for the synthesis of one or more enzymes (10, 11; Nicholson, Ph.D. thesis) but which do not sporulate in glucose-containing medium.

A number of catabolite repression-resistant mutants have been isolated which exhibit more pleiotropic phenotypes and are resistant to glucose-mediated repression of both sporulation and of a number of enzymes tested (30–32). One of these strains, GLU-47, carries a mutation, *crsA47* (*rpoD47*), in the *rpoD* (*sigA*) locus (28). *crsA47* relieves catabolite repression of sporulation and also of the enzymes acetoin dehydrogenase (32) and gluconate kinase (15). GLU-47 was transformed with pBL12 (Fig. 1). GLU-47(pBL12) was then grown in nonrepressing or repressing (1% glucose) media and assayed for β-galactosidase activity. The activity profile of β-galactosidase in cultures grown in the presence of glucose was similar to the pattern of β-galactosidase activity in nonrepressing medium (Fig. 4). The presence of glucose in the growth medium did not significantly repress *amyRi-lacZ* expression in GLU-47. Thus, *crsA47* relieves not only catabolite repression of sporulation but also repression of a gene under the control of *amyRi*. The alteration of σ^A caused by *crsA47* also restores the ability to sporulate to strains carrying *spo0E*, *spo0F*, and *spo0K* mutations (15, 17). σ^A, which is the predominant sigma factor in vegetatively growing cells, therefore plays a vital role in the initiation of sporulation and in the regulation of genes subject to glucose-mediated repression. Price and Doi (28) have suggested that the *crsA47* mutation alters σ^A so that it no longer requires certain nutritional signals or *spo0* functions to initiate transcription of sporulation-specific or sporulation-associated genes.

The activity profile of β-galactosidase from GLU-47 (pBL12) differed from the activity profile of the wild-type strains SO113(pBL4) (Fig. 2) and JH642(pBL12) (Fig. 3). In exponentially growing cultures of GLU-47(pBL12), the level

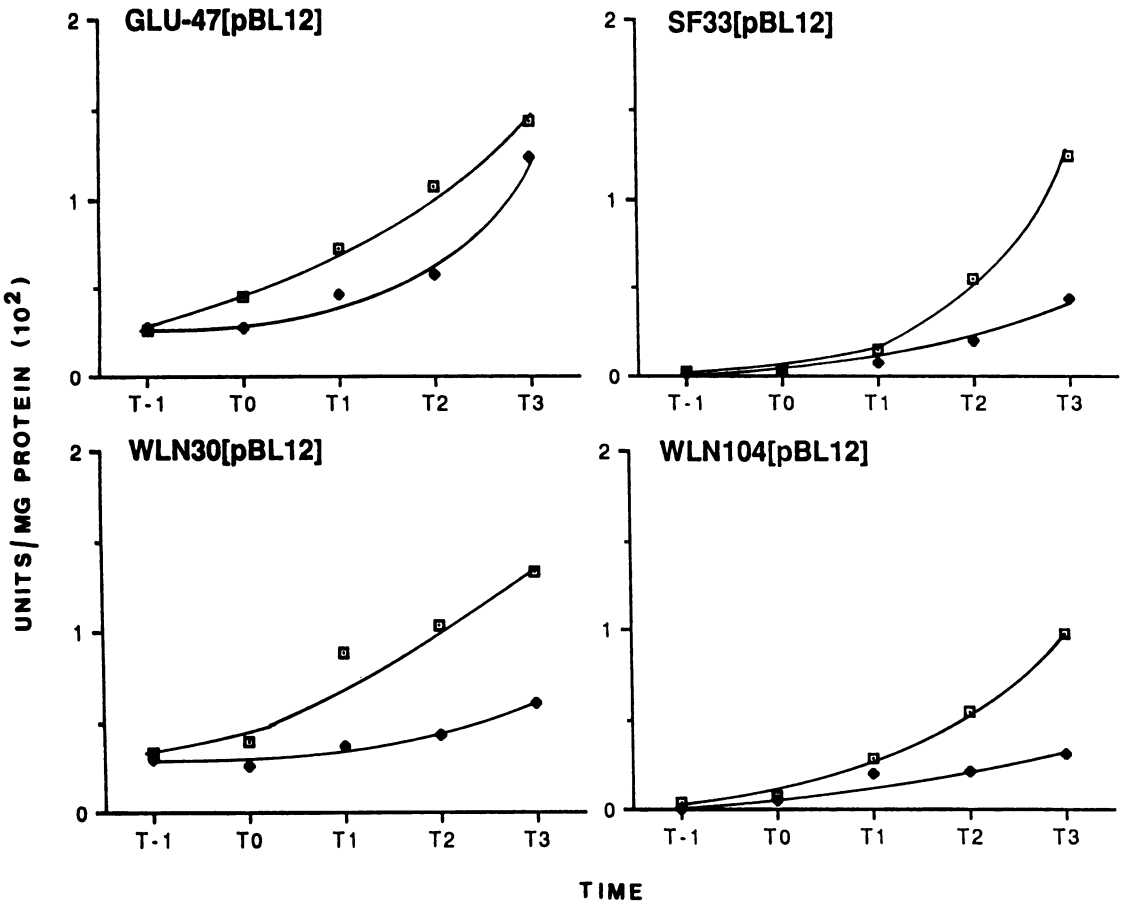

FIG. 4. β-Galactosidase activity profiles of catabolite repression-resistant strains GLU-47, SF33, WLN30, and WLN104, all harboring plasmid pBL12. Symbols and *T* values are defined in the legend to Fig. 2.

of β-galactosidase activity was two- to threefold higher than wild-type levels (Fig. 4). However, the amount of activity in late-stationary-phase cultures (T_3) of GLU-47(pBL12) was similar to wild-type levels. *amyRi-lacZ* does not appear to be temporally activated to the same extent in the mutant strain as in the wild-type strains. The mechanism of temporal activation of *amyL* is not understood. It appears to be transcribed by the major vegetative RNA polymerase, $E\sigma^A$ (16). This suggests that postexponential activation of *amyL* is not due to the appearance of a new form of RNA polymerase holoenzyme. The increased β-galactosidase activity found in exponentially growing cells harboring *crsA47* suggests a possible role for *sigA* in modulating expression of *amyL*.

Fisher and Magasanik (10) isolated a mutant, SF33 (carrying a *cdh-3* mutation), which exhibited derepressed levels of histidase, α-glucosidase, and aconitase when grown on catabolite repressing medium. SF33 showed reduced levels of pyruvate, oxaloacetate and 2-ketoglutarate similar to the levels in wild-type cells growing under nonrepressing conditions. β-Galactosidase activity profiles of an isogenic *cdh*$^+$ strain, SF32(pBL12), were similar to those of strain JH642(pBL12) under all growth conditions (data not shown). *amyRi-lacZ* expression was temporally activated (as wild type) in SF33(pBL12) (Fig. 4). However, cultures of SF33(pBL12) grown in the presence of glucose showed partially derepressed levels of β-galactosidase as compared with SF32(pBL12). The levels of β-galactosidase were four- to fivefold higher (Fig. 4) than in SF32(pBL12). The enzymes studied by Fisher and Magasanik (10) are closely involved with the glycolytic pathway; therefore, the possibility that the pleiotropic effects were simply due a defect in this pathway could not be ruled out. The fact that the *cdh-3* mutation causes derepression of *amyRi-lacZ* provides some evidence that the *cdh-3* mutation is involved in relieving catabolite repression of enzyme synthesis. However, *amyRi-lacZ* expression was only partially derepressed, which suggests that the gene product encoded by the *cdh-3* gene is one of a number of proteins involved, directly or indirectly, in mediating catabolite repression of catabolic enzyme systems.

Mutations which partially relieve glucose-mediated repression of *B. subtilis amyE* gene expression were isolated by Tn*917* insertional mutagenesis (Nicholson, Ph.D. thesis). These mutations did not affect sporulation initiation. One of these mutants, WLN30, carries the *gra-26*::Tn*917* insertion, which maps to the *aroG-argA* region of the chromosome and is likely to be an insertion within the *alsA* gene. The role of *alsA* is unclear, but it appears to be a positive regulator of *alsR-alsS*, which encodes acetolactate synthase (34). The expression of *amyRi-lacZ* in WLN30(pBL12) was fivefold higher than that of the wild type in exponentially growing cultures growing in both repressing and nonrepressing media. β-Galactosidase activity increased only two- to threefold in the stationary phase. There was partial repression of enzyme activity when WLN30(pBL12) was grown in the presence of 1% glucose. β-Galactosidase production was reduced 2- to 3-fold in cultures grown in medium containing

glucose (Fig. 4), as compared with 8- to 10-fold repression in the wild-type strain, SO113(pBL4) (Fig. 2). Expression of the *sacC* gene is also partially derepressed in a *gra-26* background (21), suggesting that the mutation has a pleiotropic effect on the expression of genes subject to catabolite repression. WLN104 is another Tn*917* insertion mutant. It carries the *gra-46*::Tn*917* insertion tentatively mapped between *metC* and *ptsI* (Nicholson, Ph.D. thesis). Glucose-mediated repression of *amyRi-lacZ* expression in this strain resulted in four- to fivefold-higher levels of β-galactosidase activity than in fully repressed wild-type strains. Temporal activation of *amyRi-lacZ* was not affected in this mutant (Fig. 4).

The *B. licheniformis amyRi* system is clearly related to other catabolite repressible systems in *B. subtilis*. *B. subtilis* regulatory proteins can efficiently regulate *amyRi*-directed expression so that the gene under its control is catabolite repressed to the same extent as in its natural host, *B. licheniformis*. Moreover, mutations in *B. subtilis* which relieve catabolite repression of *B. subtilis* systems also relieve repression of *amyRi-lacZ*. The 108-bp *cis*-acting sequence present in *amyRi* must carry information necessary to mediate catabolite repression which has been evolutionary conserved during the divergence of *B. subtilis* and *B. licheniformis*.

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